

Green Synthesis of Chitosan Nano Particles at Different Temperature for Bio-Medical Applications

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ABSTRACT

Chitosan is a semi biopolymer obtained from natural biopolymer Chitin by depolymerisation and de-acetylation. It is bio-degradable, non-toxic and an ideal candidate for pharmaceutical uses. But major bottle-neck of biomedical application of chitosan is that it is insoluble in water therefore we have reported here preparation of ionically cross linked chitosan nano material using tartarate ion as ionic cross linker by greener hydrothermal technique at different temperature and pressure to regulate particle sizes. To the best of our knowledge this is the first time we are reporting hydrothermal synthesis of chitosan nanoparticles at high pressure to regulate particle sizes. Hydro- dynamic particle sizes have been determined by DLS measurement. We have also studied fluorescence behaviour of these chitosan nanoparticles and studied their anti-bacterial activities by Alamar assay on *E Coli* and *S Aureus*.

Keywords: Chitosan nano particles (CS-NPs); De-polymerization; Hydrothermal-synthesis.

1. Introduction

Chitosan is a bio polymer obtained from chitin. It is commonly used as a wound healer [1, 2]. It is also used as gene transport vehicles [3,4], tissue culture [5,6] and textile and food packing industries. This is due to the fact that it is bio degradable having less toxicity and carcinogenicity [7,8]. The chemical structure of chitosan at neutral pH shows the presence of some $-NH_3^+$ groups. This makes it easily bind with negatively charged bio molecules [9]. Thus, muco-adhesivity property and coagulation [10,11] are due to charge interaction between CS and moccasin. This structural feature makes it a potential candidate for wound healer. Thus water soluble chitosan is essential for pharmaceutical uses. Chitosan gets protonated at acidic pH ($pH < 5.0$) and becomes soluble in water. It is reported [12-14], preparation of water soluble chitosans by chemical modifications of chitosan, attaching hydrophilic groups to its reactive $-NH_2$ group [12-14]. But chemical modification steps are often tedious and neither energy efficient nor atom efficient process. This motivates us to undertake one step green synthesis of water soluble chitosan nano particles. These water soluble chitosan particles will be an ideal bio-material for bio- medical applications [15]. When modifications are made on chitosan back bone to form a bio-polymer which may form micelle like structure [16], later may encapsulate anti-bacterial drugs.

Recently, it has been reported [17] that inherent weak fluorescence property of chitosan is enhanced upon heating due to micelle like nano structure formation. This motivates us to study exhaustively fluorescence behaviour of water soluble CS NPs. It is known [18] that chitosan nano particles can disrupt membrane and damage tumour cell. So this particle itself behaves like a potent anti-bacterial agent, we have therefore studied their anti-bacterial activities towards gram positive and gram negative bacteria.

In the present research, we have reported preparation of chitosan nano particles by hydrothermal synthesis under high temperature and pressure to regulate particle sizes. We have studied their fluorescence behaviour and

antibacterial activities towards gram positive and gram negative bacteria. To the best of our knowledge this is first time we are reporting green synthesis of chitosan nano particles (CS NPs) at different temperature and pressure.

2. Materials and methods

2.1. Method of synthesis of Chitosan nano particles

To prepare ionically cross linked Chitosan nano particle, we have used tartaric acid as cross linker. We have primarily followed our earlier protocol [19], but here we have used high pressure and temperature using controlled temperature and pressure reactor (Anton Par-hydrothermal reactor). We have taken 100 mg of low molecular weight chitosan (Sigma Aldrich) and 100 mg of L-(+) tartaric acid (Sigma Aldrich) in 10 ml DI water in a hydrothermal tube. The hydrothermal apparatus was set at 150°C for 20 minutes. The recorder associated with the apparatus records (figure 1a) temperature, pressure and time during the course of hydrothermal reaction.

After the reaction, the mixture was cooled to room temperature (27°C). The reaction mixture (S1) was a transparent fluid having a pH of 2.97. This untreated (S1) sample was filtered through 0.2 μ m filter to remove larger particles, the filtered sample (S2) which was also transparent having pH 2.93.

We have dialysed the samples for 7 days using a dialysis bag with 3.5 K Dalton molecular wt cut off, against distilled water. After dialysis, the pH raised to 6.5, this purified sample (S3) was again filtered through 0.22 μ m nylon syringe to remove any agglomerated particles to obtain final water soluble CS NPs (S4). The experiment was repeated keeping the temperatures at 180°C and 210°C. The hydrothermal curves are shown in figure 1(a) to figure 1(c).

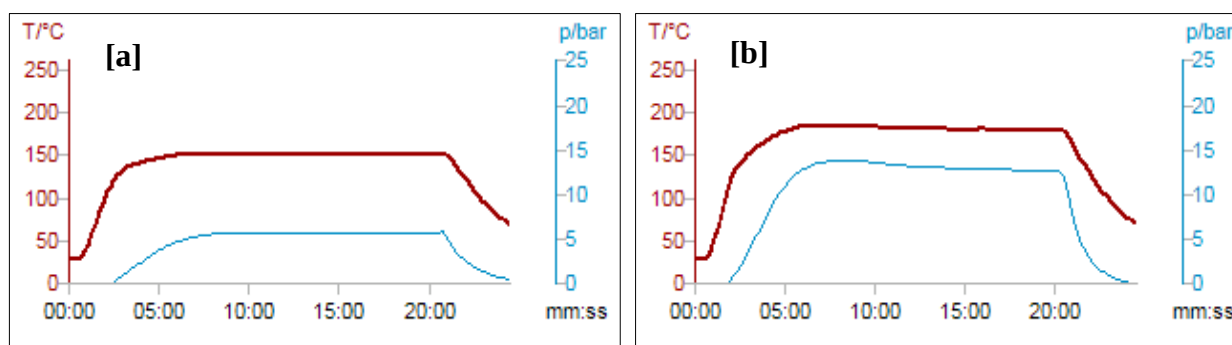
2.2. DLS method of particle size determination and fluorescence study

For DLS study water soluble sample with required dilution was taken in a DLS tube. The DLS curves are shown in figure 2a-2c. The DLS diameter of CS NPs prepared under different hydrothermal temperature is tabulated in Table 1. Fluorescence spectra of the water soluble sample at different excitation wave lengths recorded using a Nanolog Spectro fluorometer (Horiba Jobin Yvon) and shown in figure 3.

2.3. Alamar assay on E Coli and S Aureus

These test have been performed following our earlier protocol [20] and results are shown in Table 2.

3. Results and Discussions



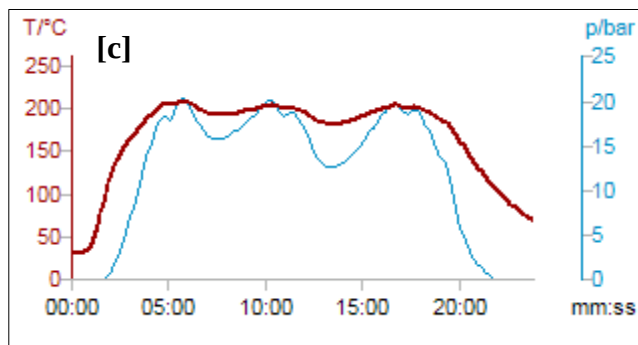


Figure 1. Hydrothermal depolymerization curve of CS-TA at 150°C (a), 180°C (b) and 210°C (c)

3.1. Hydrothermal depolymerization and DLS studies

From the figure 1, it is seen that hydrothermal depolymerization can be safely carried out at 150°C and even at 180°C, beyond that complication arises probably due to hydrothermal charring. This is supported from observed DLS diameter. It is seen (Table 1) that CS NPs formed nano particles. At 150°C and 5 bar the average nano-particle size is about ~300 nm. However at high pressure 210°C and 20 bar, it undergoes hydrothermal charring. At intermediate temperature 180°C and pressure 12 bar particle size becomes ~86 nm.

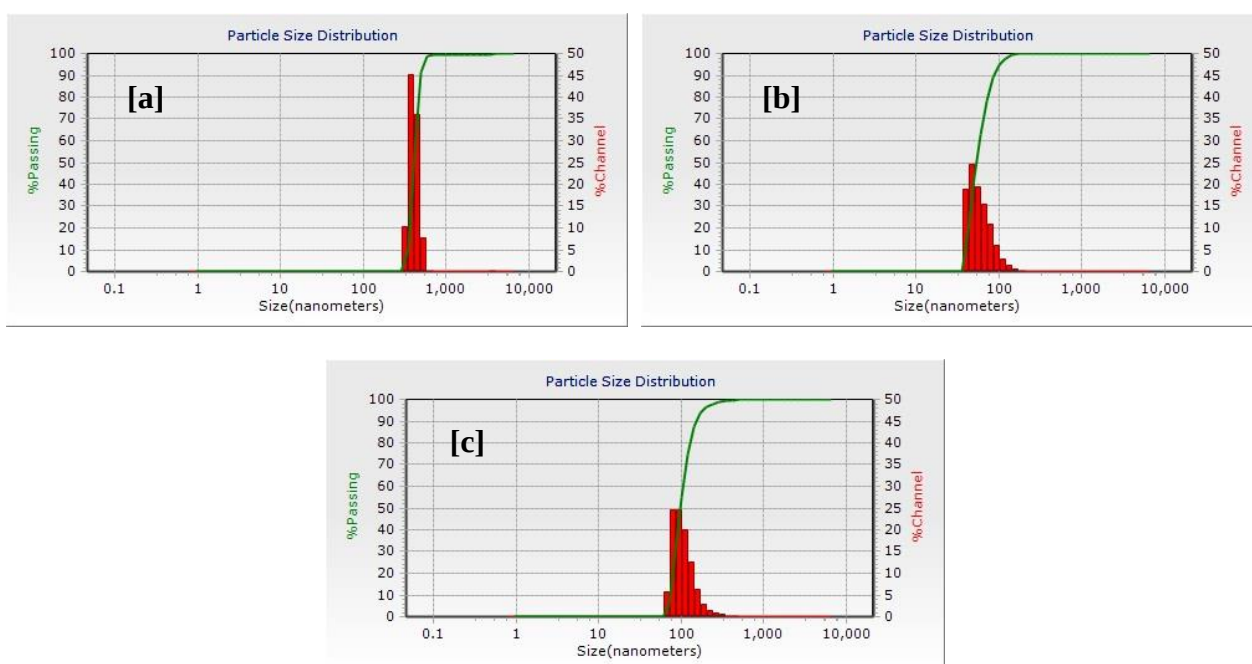


Figure 2. DLS diameter of CS-NPs particles at hydrothermal depolymerisation temperatures (a) 150°C, (b) 180°C, (c) 210°C

Table 1. Average DLS diameter of CS nano particle formed during hydrothermal de polymerization at 150°C, 180°C, 210°C

Average DLS diameter			
Temperature of hydrothermal de-polymerisation	150°C	180°C	210°C
Average DLS diameter	309 nm	85.9 nm	51.1 nm

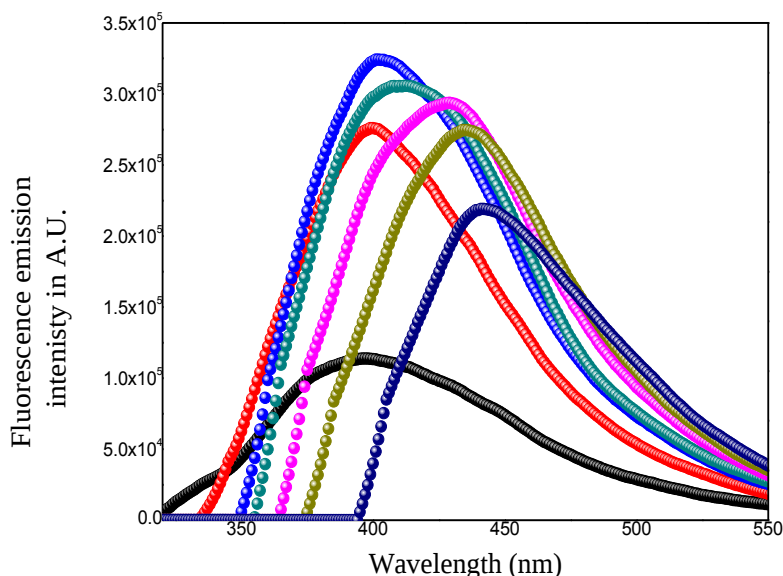


Figure 3. Fluorescence spectra of Tartaric acid de-polymerized chitosan at different excitation wavelength, λ_{ex} =300 nm (black), 320 nm (red), 335 nm (blue), 340 nm (cyan), 350 nm (pink), 360 nm (dark yellow), 380 nm (dark blue)

3.2. Fluorescence properties and antibacterial studies

From figure 3, it is noted that emission spectra depends on excited wave lengths. This clearly indicate presence of different sizes nano particles in the CS-NPs. Now the basic question is why CS-NPs exhibit fluorescence? As the chemical structure of CS contains $-NH-C=O-$ groups, it exhibits weak carbonyl fluorescence as found in poly-carbonyl compounds. Next question is why this weak carbonyl fluoresce is enhanced after nano formation. Due to compact structure of nano particles concentration of $-C=O$ groups is increased and their vibrational and rotational motion are restricted as a result non-radiative loses are minimized. Following our earlier protocol, we have studied antibacterial efficacy of *E.Coli* and *S.Aureus*.

Table 2 shows results of Alamar assay tests on *E. Coli* and *S Aureus* with two different sample of chitosan nano particles obtained at 150°C and 180°C. It is seen from the table that there is no appreciable difference in their anti-microbial activities with smaller and larger particles. This may be explained from the fact that anti-microbial activities of smaller and larger particles take place by different mechanisms. It is known chitosan exhibits promising antimicrobial activity [21]. Antibacterial efficiency of chitosan with different molecular weight and sizes have been examined. It is found that both high molecular weight CS and de-polymerized or low molecular weight CS exhibit antibacterial activities. While high molecular weight chitosan can deposit on bacteria to form a dense polymer film on the cell wall that prevent nutrient and oxygen uptake which then inhibits the growth of aerobic bacteria [21-24], low molecular weight CS binds to bacterial DNA and prevent their growth. The ability to penetrate a cell membrane and enter in the cell is also observed in this case [25-28].

Thus, de-polymerized CS acts in a better way to kill bacteria. It is worth mentioning that due to depolymerization of CS it's degree of deacetylation also decreases increasing more $-NH_2$ groups and it is the $-NH_3^+$ which plays an important role in increasing antimicrobial efficiency of CS [29]. Again, due to presence of these $-NH_3^+$ positive groups in CS, they easily bind to negatively charged bacterial surfaces [30,31].

Table 2. Almar blue test showing % of reduction of growth of bacteria due to CS nano particles

<i>S. Aureus</i> (% Reduction of colony growth) relative to control	150°C -Chitosan-400 ug/ml	180°C -Chitosan-400 ug/ml	Incubation time	Conc. of bacteria/well
	6.2	7.9	60 minutes	5X10 ⁵ CFU
<i>E. Coli</i> (% Reduction of colony growth relative to control)	150°C -Chitosan-300 ug/ml	180°C -Chitosan-300ug /ml	Incubation time	Conc. of bacteria/ well
	31.01	39.05	60 minutes	5X10 ⁵ CFU

3.3. Role of tartaric acid [TA] in the process of depolymerisation

Tartaric acid plays an important role in nano particle formation. As we have mentioned chitosan polymer chain contains positive charge centre due to the protonation of its –NH₂ groups even at neutral pH, they bind to the negatively charged tartarate in forming rigid nano particle. It should be mentioned that as the degree of protonation of CS polymer chain and degree of dissociation of tartaric acid depend on the pH of the medium, the strength of ionic cross linking or stability of CS nano particles will depend on the pH of the solution. We have noted from zeta potential [19] these CS-TA nano particles are most stable in the pH range 5.5 to 7.5.

4. Conclusions

We have demonstrated a green and cost effective method of preparation of water dispersible CS NPs nano particles of different sizes. We have optimized the preparation condition of CS NPs by carrying out the hydrothermal reaction at three different temperature and pressures which results in formation of CS nano particles of different sizes. We have examined the antimicrobial activities of these CS nano particles by Alamar blue test on *E Coli* and *S Aureus* and noted that in this case anti-microbial activities do not depend much on particle sizes. This has been explained by proposing two different mechanism of anti-bacterial activities.

Declarations

Source of Funding

This study did not receive any grant from funding agencies in the public or not-for-profit sectors.

Competing Interests Statement

Author has declared no competing interests.

Consent for Publication

The author declares that he/she consented to the publication of this study. The work have been submitted for publication with due consent of authorities of his/her institute.

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Ethical statement and Conflict of interest

The work presented here does not involve any experiment with human or animals. The work has been carried out solely by author in his/her laboratory so there is no conflict of interest in this research.

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